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71 Applicant: MERCK & CO. INC.
126, East Lincoln Avenue P.O. Box 2000
Rahway New Jersey 07065-0900(US)

72 Inventor: Bayne, Marvin L.

131 Tudor Oval
Westfield, NJ 07090(US)
Inventor: Conn, Gregory L.
1751 Essex Street
Rahway, NJ 07065(US)
Inventor: Thomas, Kenneth A., Jr.
245 Washington Avenue
Chatham Borough NJ 07928(US)

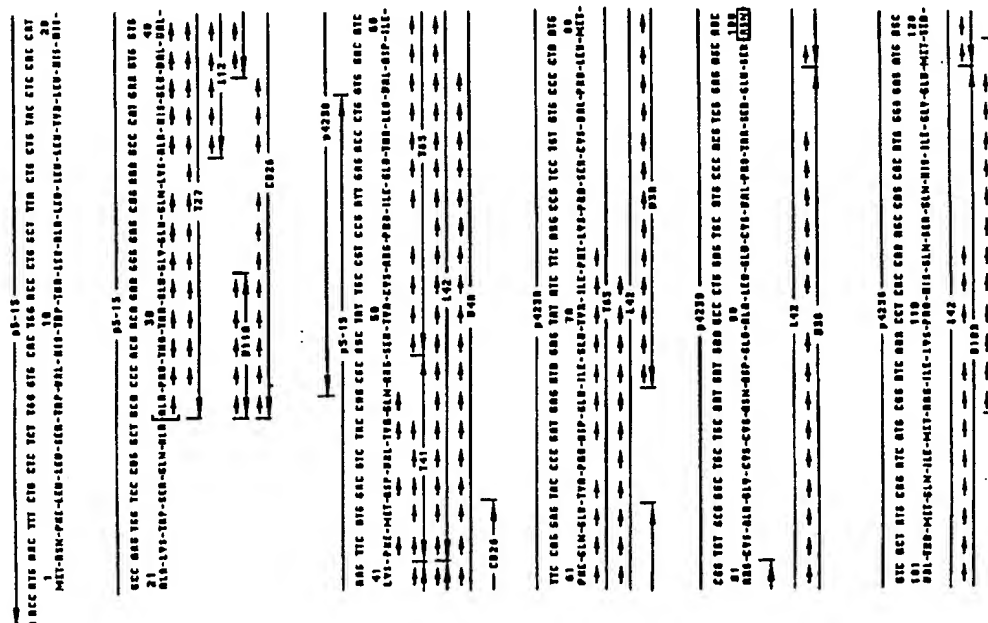
74 Representative: Hesketh, Alan, Dr. et al
European Patent Department Merck & Co.,
Inc. Terlings Park Eastwick Road
Harlow Essex, CM20 2QR(GB)

54 Purification and characterization of a glioma-derived growth factor.

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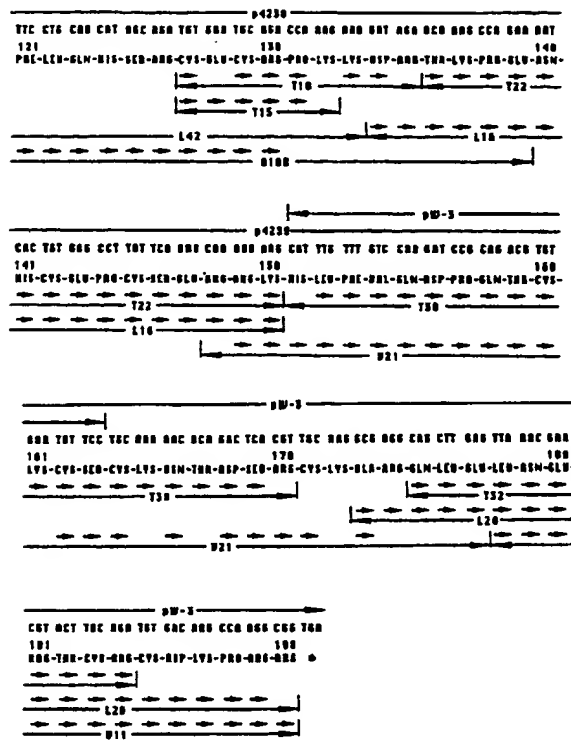
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FIGURE



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FIGURE (cont.)



PURIFICATION AND CHARACTERIZATION OF A GLIOMA-DERIVED GROWTH FACTOR

BRIEF DESCRIPTION OF THE DRAWING

The figure shows and the full length 190 amino acid residue protein translation product and its cDNA coding sequence.

BACKGROUND OF THE INVENTION

Endothelial cell mitogens are useful in promoting vascular tissue repair *in vivo*, such as blood vessel growth (angiogenesis), and vessel repair (through the replacement of damaged endothelial cells) and in stimulating endothelial cell growth on appropriate substrates for the production of blood vessels for implantation. Glioma-derived growth factor (GDGF) treatment permits the covering of synthetic polymeric vessels with non-thrombogenic vascular endothelial cells from the host animal, including man, whereby many or all of the clotting problems associated with synthetic vessel grafts are obviated. Endothelial cell stimulation with GDGF is useful for the production of vessels *in vitro* by growth of host vascular endothelial cells on tubular supports, for implantation back into the same host animal, including man, whereby immunological rejection of the implant will be obviated and the frequent limited supply of good vessels within the patient for transplant will be obviated. Tubular supports are coated *in vitro* with GDGF prior to either implantation into a host animal or coated and seeded with endothelial cells and implanted. Following implantation endothelial cells migrate into and/or grow on the artificial surface producing *in vivo* artificial vessels. As noted above GDGF can also be used for the stimulation of facilitation of blood vessel growth and repair *in vivo*, whereby the flow of blood to tissues deprived of adequate oxygen and/or other blood borne components is increased. Since GDGF can enhance vascular development and growth by the specific stimulation of vascular endothelial cells the protein can be useful in stimulating tissue repair.

OBJECTS OF THE INVENTION

It is, accordingly, an object of the present invention to provide a novel glioma-derived growth factor (GDGF). Another object is to provide a procedure for the purification of the substantially pure GDGF. A further object is to provide GDGF to stimulate endothelial cells for induction of blood vessel growth, vascular repair and the production of artificial blood vessels.

SUMMARY OF THE INVENTION

Glioma-derived growth factor is purified from the culture media used to maintain mammalian glioma cells. The protein stimulates mitogenesis of mammalian endothelial cells and is useful for the promotion of vascular development and repair. This unique growth factor is also useful in the promotion of tissue repair.

DETAILED DESCRIPTION

The present invention relates to a unique glioma derived growth factor (GDGF) which exhibits mitogenic stimulation of endothelial cells. Glioma is defined herein as any neoplasm derived from one of the various types of cells that form the interstitial tissue of the central nervous system including brain, spinal cord, posterior pituitary gland and retina. Consequently, the scope of the present invention is intended to include the unique growth factor isolated and purified from any mammalian glioma tissue or cells including cell lines. Cell lines include, but are not limited to, glioma-derived cell lines such as C6, hs 683 and GS-9L; glioblastomas such as A-172 and T98G; neuroblastomas such as IMR-32 and SK-N-MC; neurogliomas such as H4; tetromas such as XB-2; astrocytomas such as U-87 MG and U-373 MG; embryonal carcinomas and non-transformed glial or astrocyte cell lines, with GS-9L being preferred. Anterior pituitary tumor cell lines such as GH3 and Hs 199 may also be used. Although the GDGF of this invention is described as being isolated from rat cells, the same or substantially similar growth factor may be isolated from other mammalian cells, including human cells.

Glioma-derived growth factor exists in various microheterogeneous forms which are isolated from one or

more of the various cells described above. Microheterogeneous forms as used herein refer to a single gene produce, that is a peptide produced from a single gene unit of DNA, which is structurally modified at the mRNA level or following translation. Peptide and protein are used interchangeably herein. The micro-heterogeneous forms will all have equivalent mitogenic activities. Biological activity and biologically active are used interchangeably and are herein defined as the ability of GDGF to stimulate DNA synthesis in target cells including vascular endothelial cells as described below which results in cell proliferation. The modifications may take place either in vivo or during the isolation and purification process. In vivo modification results from, but is not limited to, proteolysis, glycosylation, phosphorylation, acetylation or deamidation at the N-terminus. Proteolysis may include exoproteolysis wherein one or more terminal amino acids are sequentially, enzymatically cleaved to produce microheterogeneous forms which have fewer amino acids than the original gene product. Proteolysis may also include endoproteolytic modification that results from the action of endoproteases which cleave the peptide at specific locations within the amino acid sequence. Similar modifications can occur during the purification process which also results in production of microheterogeneous forms. The most common modification occurring during purification is proteolysis which is generally held to a minimum by the use of protease inhibitors. Under most conditions one or more microheterogeneous forms are present following purification of native GDGF. Native GDGF refers to GDGF isolated and purified from cells that produce GDGF.

Glioma cells such as the rat cell line GS-9L are grown to confluence in tissue culture flasks, about 17 cm², in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with about 10% newborn calf serum (NCS). When the cells reach confluence the culture medium is removed, the cell layers washed with Ca, Mg-free phosphate buffered saline (PBS) and removed from the flasks by treatment with a solution of trypsin, about 0.1% ,and EDTA, about 0.04%. The cells, about 1×10^6 , are pelleted by centrifugation, resuspended in about 1500 ml of DMEM containing about 5% NCS and plated into a ten level cell factory (NUNC), 6,000 cm² surface area. The cells are incubated for about 48 to about 96 hours, with 72 hours preferred, at about 37° C in an atmosphere of about 5% CO₂. Following incubation the medium is removed and the cell factories are washed 3 times with PBS. Fresh culture media, about 1500 ml of about a 1:2 mixture of Ham's-F12/DMEM containing about 25 mM Hepes, about 5 µg/ml insulin, about 10 µg/ml transferrin and with or without about 1.0 mg/ml bovine serum albumin. This media is replaced with fresh media after about 24 hr and collected ever 48 hr thereafter. The collected conditioned media is filtered through Whatmen #1 paper to remove cells debris and stored at -20° C.

The GS-9L conditioned media is thawed and brought to pH 6.0 with 1 M HCl. The initial purification step consists of cation exchange chromatography using a variety of cation exchangers on a variety of matrices such as CM Sephadex, Pharmacia Mono S, Zetachrom SP and Polyaspartic Acid WCX (Nest Group) with CM Sephadex C-50 (Pharmacia) being preferred. The GDGF-containing culture media is mixed with CM Sephadex C-50 at about 1 gm per about 20 L of the conditioned media and stirred at low speed for about 24 hr at 4° C. The resin is allowed to settle and the excess liquid is removed. The resin slurry is packed into a column and the remaining culture media is removed. Unbound protein is washed from the column with 0.05 M sodium phosphate pH 6.0 containing 0.15 M NaCl. The GDGF is eluted with about 0.05 sodium phosphate pH 6.0 containing about 0.6 M NaCl.

The active fractions collected from the CM Sephadex C-50 column are further fractionated by lectin affinity chromatography for additional purification of GDGF. The lectins which may bind GDGF include, but are not limited to, lectins which specifically bind mannose residues such as concanavalin A and lens culinaris agglutinin, lectins which bind N-acetylglucosamine such as wheat germ agglutinin, lectins that bind galactose or galactosamine and lectins which bind sialic acids, with concanavalin A (Con A) being preferred. A 1.5 cm diameter column containing about 5 ml packed volume of Con A agarose (Vector Laboratories) is washed and equilibrated with about 0.05 M sodium acetate about pH 6.0 containing about 1 mM CaCl₂, about 1 mM MnCl₂ and about 0.6 M NaCl. The unbound protein is washed from the column with equilibration buffer. The GDGF is eluted with equilibration buffer containing about 0.5 M α-methyl mannoside.

The GDGF active fractions collected from the Con-A column are diluted about 1:2 with about 0.05 M sodium phosphate about pH 6.0. The sample is applied to a Polyaspartic Acid WCX cation exchange high performance liquid chromatography (HPLC) column equilibrated with the same phosphate buffer at a rate of about 0.5 ml/min. The column is eluted with a linear gradient of about 0 to 100% of about 0.75 M NaCl in the same buffer. The flow rate is maintained at about 0.75 ml/min and the fractions are collected every 60 seconds. Glioma-derived growth factor is present in fractions eluting between approximately 25 and 30 min.

The fractions containing GDGF activity eluted from the Polyaspartic Acid WCX column are loaded onto a 4.5 x 50 mm Vydac C₄ reverse phase HPLC column previously equilibrated in solvent A 0.1% trifluoroacetic acid (TFA). The column is eluted with a linear gradient over 60 min of 0 to 100% B, where B

= 33% A + 67% acetonitrile (v/v). The flow rate is maintained at 0.75 ml/min and fractions are collected every minute. The homogeneous GDGF elutes from the C₄ column under these conditions at between 29 and 32 minutes. The growth factor is eluted into about 150 µl of about 1 M Hepes buffer about pH 7.55 per 750 µl of column eluate.

5 Purity of the protein is determined by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) in 12.5% crosslinked gels using the technique of Laemmli, Nature 227: 680-684 (1970). The silver stained gels show GDGF to consist of one or more bands under non-reducing conditions with approximate molecular masses in the range of about 48,000 to about 44,000 daltons. When a sample containing the microheterogeneous forms of GDGF is separated under reducing conditions GDGF migrates as a single
10 band with a molecular mass of about 21,000 daltons.

Glioma-derived growth factor can also be extracted from non-reducing sodium dodecyl sulfate polyacrylamide gels after electrophoresis. Electrophoresis is carried out under conditions known in the art. The GDGF is eluted from the gel with buffer and the protein concentrated in a Centricon-10 centrifugal microconcentrator or an equivalent.

15 Biological activity is determined by mitogenic assay using mammalian vascular endothelial cells. Human umbilical vein endothelial (HUVE) cells are plated on gelatin coated dishes at a density of about 2000 cells per cm² in about 500 µl of Medium 199 (M199) containing about 20% heat-inactivated fetal calf serum (FCS) and about 15 mM Hepes buffer, about pH 7.55. Samples of GDGF are serially diluted in M199 plus FCS and added to the wells at the time of plating. The cells are incubated at 37° C under 5% CO₂ for 72
20 hr. After incubation the medium is removed, the cells are washed with about 250 µl of PBS, about pH 7.4 and the cells are detached with about 200 µl trypsin, about 0.1% /EDTA, about 0.04%. The cells are then counted in a hemocytometer. The concentration of GDGF which elicits a half-maximal mitogenic response is approximately 1 ng/ml.

Bovine aortic endothelial cells (BAEC) are used to evaluate thymidine incorporation following GDGF
25 treatment. BAEC are plated at a density of about 2000 cells/well in about 500 µl of DMEM containing 10% NCS and incubated at 37° C for 12 hr. The medium is removed and replaced with DMEM plus 1% FCS and the cells incubated for 48 hr. Samples of GDGF are serially diluted in DMEM containing 1% FCS and added at the end of the 48 hr period. After a further 12 hr of incubation 1.6 µCi of [methyl-³H]-thymidine (20 Ci/mmol) and 2.45 µg of labeled thymidine per ml of medium is added to the wells in 20 µl of DMEM.
30 The cells are incubated for 36 hr, washed with PBS and lysed with 250 µl of a solution of 2 g sodium carbonate and 0.4 g NaOH per 100 ml. The amount of incorporation of radiolabel into cellular DNA is determined by scintillation counting.

Samples of the native dimeric GDGF, 48 kilodalton (kDa), 46 kDa and 44 kDa, and a sample of the reduced and carboxymethylated monomer (21 kDa) are hydrolyzed for about 24 hr in constant boiling 6N
35 HCl and their amino acid compositions determined using the Waters Pico Tag system. The amino acid composition is shown in Table 2.

A sample of purified GDGF is reduced in about 0.1 M Tris pH about 9.5 with about 0.1% EDTA, about 6 M guanidinium chloride and about 20 mM dithiothreitol for about 2 hr at about 50° C. The reduced protein is carboxymethylated by the addition of about 9.2 µM of unlabelled and 2.8 µM of ¹⁴C-iodoacetic acid in
40 about 0.7 M Tris about pH 7.8 containing about 0.1% EDTA and about 6 M guanidinium chloride. The protein is carboxymethylated for about 1 hr at room temperature. The protein was isolated by reverse phase HPLC as described above. A sample of the reduced and carboxymethylated monomer was treated with either the protease trypsin which cleaves polypeptides on the C-terminal side of lysine and arginine residues or Lys C which cleaves polypeptides on the C-terminal side of lysine by procedures well known in
45 the art. The peptides are isolated by reversed phase HPLC (RP-HPLC). The amino acid sequences of the isolated peptides are determined using the Edman degradation in the ABI gas phase sequencer in conjunction with the ABI 120 A on line phenylthiohydantoin analyzer following manufacturer's instructions. The amino acid sequences are shown in Table 3.

Reduced and carboxymethylated GDGF is dried and solubilized in about 0.7 M Tris, about pH 7.8,
50 about 6 M guanidinium chloride containing about 0.1% EDTA. V8 protease is added in an ammonium bicarbonate buffer, about pH 8.0 and the mixture incubated for about 48 hr at about 37° C. The protease cleaves predominantly on the carboxyl terminal side of glutamic acid residues. The resulting polypeptides were resolved by C₁₈ RF-HPLC as above.

Following reduction and carboxymethylation of GDGF the protein solution is adjusted to a pH of about
55 6.8 with 6 N HCl and dithiothreitol is added to a final concentration of 2 M for reduction of any methionine sulfoxide to methionine residues. After about 20 hr of reduction at about 39° C the protein is repurified by C₄HPLC. The product is dried and cleaved on the carboxyl terminal side of methionine residues by 200ml of 40 mM cyanogen bromide in about 70 % (v/v) formic acid under an argon atmosphere at about 20° C for

about 24 hr in the dark. The cleavage products are resolved by C₁₈ RP-HPLC. The amino acid sequence is shown in Figure 1.

The full length 190 amino acid residue protein translation product and its cDNA coding sequence are shown in Fig. 1. The mature amino terminus begins at residue 27, immediately following a typical hydrophobic secretory leader sequence. A single potential N-glycosylation site exists at Asn₁₀₀. Most (143 amino acid residues) of the 164 residues of the reduced and carboxymethylated mature subunit including the amino terminus and HPLC reversed phase-purified products of tryptic (T), Lys-C (L), *Staphylococcus aureus* V8 protease (V8) and cyanogen bromide (CB) cleavages, were determined by direct microsequencing (Applied Biosystems 470A) using a total of 5 mg of protein. All residues identified by amino acid sequencing are denoted by arrows pointing to the right either directly beneath the listed sequence following the bracket at residue 27 for the amino terminal determination of the whole subunit or, for residues identified from the polypeptide cleavage products, above the double-headed arrows spanning the length of the particular polypeptide. One listed pair of polypeptides, V18A and V18B, was sequenced as a mixture and, therefore, are only confirmatory of the cDNA-deduced amino acid sequence, see figure.

The full length coding region was determined from three sets of overlapping cDNA clones. Degenerate oligonucleotide primers based on the amino acid sequences Phe-Met-Asp-Val-Tyr-Gln from polypeptide L42 (residues 42-47) and Cys-Lys-Asn-Thr-Asp from polypeptide T38 (residues 164-168) were used to PCR amplify the central region of the cDNA for GDGF following the procedure of Saiki et al., *Science* 230; 1350-1354 (1985). A single band migrating at 420 bp was gel purified, digested with *Sal*I, ligated into pGEM3Zf(+) and sequenced. The nucleotide sequence obtained (p4238) was used to design antisense and sense PCR primers to amplify the 5' and 3' ends of the cDNA according to the protocol described by Frohman et al. *Proc Natl. Acad. Sci. USA* 85:8998-9002 (1988). These 5' and 3' clones are denoted p5-15 and pW-3, respectively. Regions of complete DNA sequences, excluding the primers, determined for each set of clones are indicated by double-headed arrows above the nucleotide sequence.

It is intended that the nucleotide sequence for glioma-derived growth factor be interpreted to include all codons that code for the appropriate amino acid in the sequence for glioma-derived growth factor, as indicated by the degeneracy of the genetic code. It is further intended that the nucleotide sequence and the amino acid sequence for glioma-derived growth factor include truncated genes or proteins which result in a protein which exhibits biological activity similar to glioma-derived growth factor.

Expression of the GDGF gene is accomplished by a number of different promoter-expression systems and in a number of different cells. Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of recombinant DNA sequences or genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express genes in a variety of hosts such as bacteria, blue-green algae, yeast cells, insect cells, plant cells and animal cells.

The ability of GDGF to stimulate the division of vascular endothelial cells makes this peptide in all microheterogeneous forms useful as a pharmaceutical agent. The protein as used herein is intended to include all microheterogeneous forms as previously described. The protein can be used to treat wounds of mammals including humans by the administration of the novel protein to patients in need of such treatment.

The novel method for the stimulation of vascular endothelial cells comprises treating a sample of the desired vascular endothelial cells in a nutrient medium with mammalian GDGF, preferable human or rat, at a concentration of about 1-10 ng/ml. If the vascular endothelial cell growth is conducted *in vitro*, the process requires the presence of a nutrient medium such as DMEM or a modification thereof and a low concentration of calf or bovine serum such as about 0 to 2% by volume. Preservatives such as antibiotics may also be included, these are well known in the art.

The novel growth factor of this invention is useful for the coverage of artificial blood vessels with vascular endothelial cells. Vascular endothelial cells from the patient would be obtained by removal of a small segment of peripheral blood vessel or capillary-containing tissue and the desired cells would be grown in culture in the presence of GDGF and any other supplemental components that might be required for growth. After growth of adequate numbers of endothelial cells in culture to cover a synthetic polymeric blood vessel the cells would be plated on the inside surface of the vessel which is then implanted in the patient. Alternatively, tubular supports are coated *in vitro* with GDGF prior to implantation endothelial cells migrate into and grow on the artificial surface. Prior coating of the artificial vessel either covalently or noncovalently, with proteins such as fibrin, collagen, fibronectin or laminin would be performed to enhance attachment of the cells to the artificial surface. The cell-lined artificial vessel would then be surgically implanted into the patient and, being lined with the patient's own cells, would be immunologically compatible. The non-thrombogenic endothelial cell lining should decrease the incidence of clot formation on the surface of the artificial vessel and thereby decrease the tendency of vessel blockage or embolism elsewhere.

The novel protein is also useful for the production of artificial vessels. Vascular endothelial cells and smooth muscle cells from the patient would be obtained and grown separately in culture. The endothelial cells would be grown in the presence of GDGF as outlined above. The smooth muscle would be grown in culture by procedures well known in the art. A tubular mesh matrix of a biocompatible polymer (either a synthetic polymer, with or without a coating of proteins, or a non-immunogenic biopolymeric material such as surgical suture thread) would be used to support the culture growth of the smooth muscle cells on the exterior side and vascular endothelial cells on the interior surface. Once the endothelial cells form a confluent monolayer on the inside surface and multiple layers of smooth muscle cells cover the outside, the vessel is implanted into the patient.

The novel peptide can also be used for the induction of tissue repair or growth. The pure GDGF would be used to induce and promote growth of tissue by inducing vascular growth and /or repair. The peptide can be used either topically for tissue repair or intravascularly for vascular repair. For applications involving neovascularization and healing of surface wounds the formulation would be applied directly at a rate of about 10 ng to about 1 mg/cm²/day. For vascular repair GDGF is given intravenously at a rate of about 1 ng to about 100 µg/kg/day of body weight. For internal vascular growth, the formulation would be released directly into the region to be neovascularized either from implanted slow release polymeric material or from slow release pumps. The release rate in either case is about 100 ng to about 100 µg/day/cm³.

For non-topical application the GDGF is administered in combination with pharmaceutically acceptable carriers or diluents such as, phosphate buffer, saline, phosphate buffered saline, Ringer's solution, and the like, in a pharmaceutical composition, according to standard pharmaceutical practice. For topical application, various pharmaceutical formulations are useful for the administration of the active compound of this invention. Such formulations include, but are not limited to, the following: ointments such as hydrophilic petrolatum or polyethylene glycol ointment; pastes which may contain gums such as xanthan gum; solutions such as alcoholic or aqueous solutions; gels such as aluminum hydroxide or sodium alginate gels; albumins such as human or animal albumins; collagens such as human or animal collagens; celluloses such as alkyl celluloses, hydroxy alkyl celluloses and alkylhydroxyalkyl celluloses, for example methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; polyoxamers such as Pluronic® Polyols exemplified by Pluronic® F-127; tetronics such as tetriconic 1508; and alginates such as sodium alginate.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1

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Preparation of Medium Conditioned by GS-9L Cells

GS-9L cells were grown to confluence in 175 cm² tissue culture flasks in Dulbecco's Modified Eagle's Medium/10% newborn calf serum (DMEM/NCS). At confluence the medium was decanted from the flasks, the flasks were washed with calcium and magnesium free phosphate buffered saline (PBS) and the cells are removed by treatment with a 1X solution of trypsin/EDTA. The cells (1 x 10⁸) were pelleted by centrifugation, resuspended in 1500 ml. of DME/5% NCS and plated into a ten level (6000 cm² surface area) cell factory (NUNC). After 72 hours incubation at 37° C in a 5% CO₂ atmosphere the medium was decanted and the cell factories were washed 3X with PBS. The cells were refed with 1500 ml of a 1:2 mixture of Ham's F-12/DMEM containing 25 mM Hepes, 5 micrograms/ml insulin, 10 micrograms/ml transferrin and 1.0 mg/ml bovine serum albumin. This medium was changed with fresh F-12/DMEM after 24 hours and collected every 48 hours after that. The conditioned medium was filtered through a Whatman #1 paper to remove cell debris and stored frozen at -20°C.

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EXAMPLE 2

Carboxymethyl-Sephadex Chromatography

GS-9L conditioned medium, from Example 1, was thawed and brought to pH 6.0 with 1M HCl. One gram of CM Sephadex C-50 cation exchange resin pre-equilibrated in PBS adjusted to PH 6.0 with 1N HCl is added to 20 liters of conditioned medium. The mixture was stirred at low speed for 24 hours at 4° C. The resin was then allowed to settle and the medium is siphoned off. The remaining resin slurry was packed
 5 into a 3.0 cm diameter column and any remaining medium is allowed to drain off. Unbound protein was washed off the column with 0.05 M sodium phosphate pH 6.0 containing 0.15 NaCl. Glioma-derived growth factor activity was eluted from the column with a subsequent wash of 0.05 M sodium phosphate, pH 6.0, containing 0.6 M NaCl.

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EXAMPLE 3

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Concanavalin A Affinity Chromatography

A 1.5 cm diameter column containing 5 ml. packed volume of Con A agarose (Vector Laboratories) is washed and equilibrated with 0.05M NaAcetate pH 6.0 containing 1mM CaCl₂ , 1mM MnCl₂ and 0.6M NaCl.
 20 The CM Sephadex C-50 eluate containing the GDGF activity, from Example 2, was applied to the column and unbound protein was removed from the column with a wash of equilibration buffer. GDGF activity was eluted from the column by a wash of equilibration buffer containing 0.5M α -methyl mannoside.

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EXAMPLE 4

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Polyaspartic Acid WCX Cation Exchange Chromatography

A 4.5 mm x 250 mm Polyaspartic Acid WCX cation exchange HPLC column (Nest Group) was equilibrated in 0.05M sodium phosphate, pH 6.0. The GDGF activity eluted from the Con A agarose, from Example 3, was applied to the Polyaspartic Acid column at 0.5 ml/min. The column was then eluted with a
 35 linear gradient of 0 to 100% B buffer over 30 minutes where A = equilibration buffer and B = A containing 0.75M NaCl. The flow rate was maintained at 0.75 ml/min and fractions are taken every minute. GDGF activity was found in the fractions eluted between 25 and 30 minutes.

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EXAMPLE 5

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Reverse Phase HPLC Chromatography

The fractions containing GDGF activity eluted from the Polyaspartic Acid column, Example 4, were loaded onto a 4.5 x 50 mm Vydac C4 reverse phase HPLC column which was previously washed with 0.1% trifluoroacetic acid (TFA) solution. The column was eluted with a linear gradient over 60 minutes of 0 to
 50 100% B where A = 0.1% TFA and B = 33% A, 67% acetonitrile. The flow rate was maintained at 0.75 ml/min and fractions are collected every minute. The homogeneous GDGF eluted from the C4 column under these conditions between 29 and 32 minutes into the gradient. The growth factor was eluted into 150 μ l of 1M HEPES buffer, pH 7.55, per 750 μ l of column eluate in order to maintain mitogenic activity SDS-PAGE analysis of the protein on silverstained gels showed GDGF to consist of 3 bands under non-reducing
 55 conditions of apparent molecular mass 48,000, 46,000 and 44,000 daltons. Apparent molecular mass was calculated from calibrated protein standards of known molecular mass. All procedures are well known in the art. Under reducing conditions GDGF appears as a single band of protein with a molecular weight of 21,000.

EXAMPLE 6Mitogenic Assays

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Human umbilical vein endothelial cells were plated on gelatin-coated 48 well tissue culture dishes at a density of 2000 cells/well in 500 μ l of Medium 199 containing 20% heat inactivated fetal calf serum (FCS) and 15 mM Hepes buffer. Samples of GDGF, from Examples 1-5, to be assayed were serially diluted in M199/fetal calf serum and added to the wells at the time of plating. The culture plates are incubated at 37° C in 5% CO₂ for 72 hours. After incubation the medium was removed from the cells, which are then rinsed with 250 μ l of PBS and the cells are detached with 200 μ l of trypsin (0.1%)/EDTA (0.04%). The cells were then counted in a hemocytometer.

Bovine aortic endothelial cells were plated in 48 well plates at a density of 2000 cells/well in 500 microliters of DMEM/10% NCS and incubated at 37° C for 12 hours. The medium was removed from the plates and replaced with DMEM/1% heat inactivated FCS and the cells are again incubated for 48 hours. Samples of GDGF, from Examples 1-5, to be assayed were serially diluted in DMEM/1%FCS and added to the plates at the end of this 48 hour period. After a further 12 hours of incubation 1.6 μ Ci of [methyl-³H]-thymidine (20 Ci/mmol) and 2.45 μ g of unlabeled thymidine per milliliter of medium was added to the wells in 20 μ l of DMEM. The plates were incubated again for 36 hours, the medium is removed, the cell layers were washed with PBS and the cells were lysed with 250 μ l of a solution of 2 grams sodium carbonate and 0.4 grams NaOH per 100 ml. The amount of incorporation of radiolabel into DNA was then determined by scintillation counting.

The concentration of GDGF which elicited a half-maximal mitogenic response in HUVE cells was approximately 1 ng/ml. The glycosaminoglycan heparin, which is required in these assays at a level of 10-100 μ g/ml to promote a response to a positive control, acidic fibroblast growth factor, does not enhance mitogenic stimulation of these cells by GDGF.

Utilizing the mitogenic assay for BALB/C 3T3 fibroblasts as described by Linemeyer et al., Bio/Tech. 5: 960-965 (1987), GDGF was unable to stimulate a mitogenic response in 3T3 cells. Employing the biochemical procedures and analytical techniques described above, the stepwise purification is summarized in Table 1.

TABLE 1

35

Purification of Glioma-Derived Growth Factor ¹				
Purification Step	Protein (mg)	Units (mg/ml)	Sp. Activity (units/mg)	Purification Factor
Conditioned Medium	2.5 x 10 ³	20,000	8	1
CM Sephadex C-50	16	12,000	7.5x10 ²	94
Con A	1.2	4,000	3.3x10 ³	417
Polyaspartic Acid WCX	1.1 x 10 ⁻²	1,5000	1.4 x 10 ⁵	17,500
C ₄ HPLC	1.0 x 10 ⁻³	1,000	1.0 x 10 ⁶	125,000

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¹ Based on 5 L of conditioned medium

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EXAMPLE 7

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Amino Acid Composition And Sequence

Both samples of the native dimeric GDGF and a sample of the reduced and carboxymethylated

monomer have been hydrolyzed and their amino acid compositions determined using the Waters PicoTag system. Both samples of the native dimeric GDGF and a sample of reduced and carboxymethylated monomer. (generated by reduction and carboxymethylation of GDGF using the protocol listed below for the amino acid sequence determination) were hydrolyzed in constant boiling HCl containing 1% phenol (Pierce) for 24 hr at 110° C. The hydrolysate was subsequently derivatized with phenylisothiocyanate to yield the phenylthiocarbamyl amino acids (PTC). The PTC amino acids were resolved by reverse phase chromatography on a Waters PicoTag amino acid analysis HPLC column and quantitated by comparison of their absorbances at 254 nm to standard PTC amino acids of known amount. The exact protocol is detailed in Waters PicoTag Amino Acid Analysis System manual No. 88140, February, 1986. The amino acid composition is based on a molecular mass of 35,000 daltons for the dimeric form of GDGF minus oligosaccharide chains. The amount of oligosaccharide present on GDGF was determined by comparing the molecular mass of GDGF in reducing SDS-PAGE before and after removal of oligosaccharides with the enzyme Endoglycosidase H. Twenty-five ng of pure GDGF in 0.05 M sodium citrate, pH 6.0, 0.01% SDS and 0.1 M beta-mercaptoethanol, was treated with 20 mU/ml of Endoglycosidase H for 6 hr at 37° C. The product was subsequently electrophoresed in 12.5% SDS-PAGE and silver stained to determine the alteration in relative mobility due to oligosaccharide removal in order to establish the effect of the oligosaccharide sidechains on protein molecular mass estimates. The results are shown in the following table.

TABLE 2

Amino Acid Composition Of Dimeric GDGF	
Amino Acid	Units
Asp	20
Glu	44
Ser	24
Gly	10
His	10
Arg	24
Thr	18
Ala	12
Pro	28
Tyr	8
Val	16
Phe	8
Met	8
Cys	28
Ile	12
Leu	14
Lys	18

Tryptophan has not been determined. Cystine was estimated from the level of incorporation of ¹⁴C-iodoacetic acid into the reduced and carboxymethylated monomer.

A 4 µg sample of native dimeric GDGF was reduced in 0.1 M Tris, pH 9.5, with 0.1% EDTA, 6 M guanidinium chloride and 20 mM dithiothreitol for 2 hr at 50° C. The reduced protein was carboxymethylated by the addition of 9.2 µM of unlabeled and 2.8 µM of ¹⁴C-iodoacetic acid in 0.7 M Tris pH 7.8 containing 0.1% EDTA and 6 M guanidinium chloride. The protein was carboxymethylated for 1 hr at room temperature. The protein was subsequently reisolated by C₄ RP-HPLC as described above. A sample of the reduced and carboxymethylated monomer was treated with either the protease trypsin or Lys C. For trypsin, reduced and carboxymethylated GDGF was treated with a 1:100 ratio by weight of tosylphenylchloroketone-trypsin to GDGF in 0.1 M ammonium bicarbonate, pH 8.3 for 6 hr at 37° C. The resultant peptide fragments were resolved by chromatography on a 4.5 mm x 25 cm C₁₈ reverse phase

column. Peptides were eluted with a linear gradient over 3 hr of 0-100% B where A = 0.1 TFA and B = 0.1% TFA containing 67% acetonitrile. For Lys C cleavage all conditions were identical except that the buffer used was 0.1 M Tris, pH 8.5, a 40:1 ratio of GDGF to Lys C was maintained and the GDGF and Lys C mixture was incubated for 14 hr. The amino acid sequences of the isolate peptides were then determined using Edman degradation in an ABI gas phase sequenator in conjunction with the ABI 120 A on line phenylthiohydantoin analyzer. (Applied Biosystems Int.). The peptide sequences are shown in the following table.

TABLE 3
Amino Acid Sequences
Trypsin

T22

Thr-Lys-Pro-Glu-Asn-His-Cys-Glu-Pro-Cys-Ser-Glu-Arg-Arg-Lys

T27

Xxx^a-Xxx-Thr-Thr-Glu-Gly-Glu-Gln-Lys-Ala-His-Glu-Val-Val-Lys

T38

His-Leu-Phe-Val-Gln-Asp-Pro-Gln-Thr-Cys-Lys-Cys-Ser-Cys-Lys-Asn-Thr-Asp

T40

Phe-Met-Asp-Val-Tyr-Gln-Arg

Lys C

L16

Asp-Arg-Thr-Lys-Pro-Glu-Asn-His-Cys-Glu-Pro-Cys-Ser-Glu-Arg-Arg-Lys

L20

Ala-Arg-Gln-Leu-Glu-Leu-Asn-Glu-Arg-Thr-Cys-Arg-Cys-Asp-Lys-Pro-Arg

L23

His-Leu-Phe-Val-Gln-Asp-Pro-Gln-Thr-Cys-Lys

^a denotes an unidentified amino acid residue.

The amino acid sequences of the isolated peptides illustrates the unique nature of GDGF.

GDGF was stored at 4 °C in the aqueous trifluoroacetic acid (TFA)/acetonitrile mixture used to elute the homogeneous protein in reversed phase C₄ HPLC chromatography at the final stage of the purification protocol previously described. Aliquots of the purified protein (1-2 µg) were vacuum evaporated to dryness is acid-washed 10 x 75 mm glass tubes and reduced for 2 hours at 50 °C in 100 µl of 0.1 M Tris buffer, pH 9.5, and 6 M guanidinium chloride containing 0.1% EDTA and 20 mM dithiothreitol (Calbiochem, Ultrol grade) under an argon atmosphere. The reduced protein was subsequently carboxymethylated for 1 hour at 20 °C by the addition of 100 µl of 0.7 M Tris, pH acid and 50 µCi of iodo[2- ¹⁴C]acetic acid (17.9 mCi/mmol, Amersham). After completion of the carboxymethylation, the mixture was loaded directly onto a 4.6 mm x 5.0 cm Vydac C₄ column which had been pre-equilibrated in 0.1% TFA. The reduced and carboxymethylated protein was repurified by elution with a 30 minute linear gradient of 0 to 67% (v/v) acetonitrile in 0.1% TFA at a flow rate of 0.75 ml/min and stored in this elution solution at 4 °C

Reduced and carboxymethylated GDGF (725 ng) was dried by vacuum evaporation in an acid-washed 10 x 75 mm glass tube. TPCK-treated bovine pancreatic trypsin (30 ng, Worthington) was added in 200 µl of 0.1 M ammonium bicarbonate, pH 8.3. The substrate protein was digested at 37 °C for 6 hours and the resulting polypeptides loaded onto a 4.6 mm x 25 cm Vydac C₁₈ reversed phase HPLC column equilibrated in 0.1% TFA. Polypeptides generated by proteolysis on the carboxyl terminal side of arginine and lysine residues were fractionated by elution with a 2 hour linear gradient of 0-67% acetonitrile in 0.1% TFA at a flow rate of 0.75 ml/min at 20 °C. Individual peaks were of 0.75 ml/min and stored in this elution solution at 4 °C.

Reduced and carboxymethylated GDGF (925 ng) was dried by vacuum evaporation in an acid-washed 10 x 75 mm glass tube. Lys C protease (50 ng, Boehringer Mannheim), an enzyme that cleaves on the carboxyl terminal side of lysine residues, was added in 50 µl of 0.1 M Tris, pH 8.5, 1 mM EDTA. The substrate protein was digested at 37 °C for 8 hours and the resulting polypeptides resolved by reversed phase HPLC chromatography on a C₁₈ column as described above.

Reduced and carboxymethylated GDGF (1.1 µg) was dried and solubilized in 5 µl of 0.7 M Tris, pH 7.8, 6 M guanidinium chloride containing 0.1% EDTA. V8 protease (65 ng, Miles Labs) was added in 65 µl of 0.1 M ammonium bicarbonate, pH 8.0, 0.1% EDTA and the mixture was incubated for 48 hours at 37 °C. Under these digestion conditions, this protease cleaves predominantly on the carboxyl terminal side of glutamic acid residues. The resulting polypeptides were resolved by reversed phase HPLC chromatography on a C₁₈ column as described above.

Following reduction and carboxymethylation of GDGF (1.3 µg) the protein solution was adjusted to pH 6.8 with 6 N HCl and dithiothreitol was added to a final concentration of 2 M for reduction of any methionine sulfoxide to methionine residues. Following 20 hours of reduction at 39 °C the protein was repurified on a C₄HPLC reversed phase column was previously described. The product was dried in a 10 x 75 mm glass tube and cleaved on the carboxyl terminal side of methionine residues by 200 µl of 40 mM cyanogen bromide in 70% (v/v) formic acid under an argon atmosphere at 20 °C for 24 hours in the dark. The cleavage products were resolved by reversed phase HPLC chromatography on a C₁₈ column as previously described.

EXAMPLE 8

PCR Amplification, Cloning and Sequencing of P4238

Two degenerate oligonucleotides were synthesized in order to amplify the cDNA encoding the peptide sequences of GDGF between lysC fragment 42 and tryptic fragment 38. These oligonucleotides were:

42.2 5' TTTGTCGACTT[TC]ATGGA[TC]GT[N]TA[TC]CA3'
T383'B 5'

CAGAGAATTCGTCGACA[AG]TC[N]GT[AG]TT[TC]TT[AG]CA3'

where N = ACGT

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RNA was isolated from GS9L cells using the Fast Track RNA isolation kit from Invitrogen and the protocol provided. First strand cDNA synthesis was performed as follows:

- 1 microgram of GS9L RNA was annealed to 1 µg of adapter primer TA17, 5' GACTCGAGTCGACATC-
5 GATTTTTTTTTTTTTTTT 3', by incubating in a volume of 10 µl of 70C for 5 min. followed by cooling to
room temperature. To this reaction was added
3.0 µl water
2.5 µl 10X buffer (500mM Tris-HCl pH 8.3,
750mM KCl, 100mM MgCl₂, 5mM spermidine)
10 2.5 µl 100mM DTT
2.5 µl 10 mM each dATP, dGTP, dCTP, dTTP
0.6 µl 15units RNasin
2.5 µl 40mM Na pyrophosphate
1.5 µl 15 units reverse transcriptase
15 The reaction was incubated at 42° C for 1 hour, then diluted to 1ml in 10mM Tris-HCl 1mM EDTA pH
7.5
PCR Reactions
Primary reaction 100µl
10µl 10X buffer from Perkin Elmer Cetus GeneAmp kit
20 16µl 1.25mM each stock of dATP, dCTP, dGTP, and dTTP
2µl first strand GS9L cDNA
2µl 50pMoles L42.3
2µl 50pMoles T383'B
0.5µl 2.5units Amplitaq DNA polymerase
25 67.5µl water
Reaction conditions 40 cycles of 94C 1'; 50C 2'30"; 72C 2'
Prep scale secondary reaction
10µl 10X buffer
160µl 1.25mM each stock of dATP, dCTP, dGTP, and dTTP
30 10µl primary PCR reaction
20µl 500pMoles L42.2
20µl 500pMoles T383'B
5µl 25 units Amplitaq DNA polymerase
685 µl water
35 Reaction conditions 94C 1'; 55C 2'; 72C 2' 30 cycles
The PCR product was concentrated by Centricon 30 spin columns, purified on a 1% agarose gel, and
digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+).
The ligation mix was used to transform E. coli XL-1 blue. Plasmid DNA was isolated from white
transformants and sequenced by the dideoxy chain termination method.

EXAMPLE 9

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PCR Amplification, Cloning and Sequencing of pW-3

- Based on the sequence obtained from the p4238 clones, two specific PCR primers were synthesized;
50 oligo307 5' TTTGTCTGACTCAGAGCGGAGAAAGC 3' and oligo289 5' TTTGTCTGACGAAAATCACTGTGAGC
3'. These primers were used in combination with oligoA17 5' GACTCGAGTCGACATCG3' to amplify the
cDNA encoding the COOH terminus of GDGF using the 3' RACE technique described by Frohman et al.,
PNAS 85: 8998-9002 (1988).
PCR reactions
55 Primary reaction 100µl
10µl 10X buffer from Perkin Elmer Cetus GeneAmp kit
18µl 1.25mM each stock of dATP, dCTP, dGTP, and dTTP
0.35µl first strand GS9L cDNA

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- 2μl 50pMoles 289
0.5μl 2.5 units Amplitaq DNA polymerase
67.15μl water
Reaction conditions 94C 1'; 58C 2'; 72C 2' 10 cycles then added 50pMoles A17, then 1 cycle of 94C 1';
5 58C 2'; 72C 40' followed by 40 cycles 94C 1'; 58 2'; 72C 2'.
Prep Scale secondary reaction
60μl 10X buffer
108μl 1.25mM each stock of dATP, dCTP, dGTP, and dTTP
24μl primary PCR reaction
10 12μl 300pMoles 307
12μl 300pMoles A17
3μl 15 units Amplitaq DNA polymerase
381μl water
15 Reaction conditions 94C 1'; 58C 2'; 72C 2' 30 cycles
The PCR product was purified on a 1% agarose gel and digested with restriction endonuclease Sall.
The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform E.
coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain
termination method.

EXAMPLE 10

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PCR Amplification, Cloning and Sequencing of p5-15

- Based on the sequence of p4238 clones, two specific PCR primers were synthesized; oligo113
30 5' TTTGTCGACAACACAGGACGGCTTGAAG 3' and oligo74 5' TTTGTCGACATACTCCTGGAAGATGTCC
3'. These primers were used in combination with oligoA17 5' GACTCGAGTCGACATCG 8' to amplify the
cDNA encoding the amino terminus of GDGF using the 5' RACT technique described by Frohman et al.,
supra. Oligo 151 was synthesized in order to specifically prime GDGF cDNA from GS9L RNA. Oligo 151 is
5' CTTTCATCATTGCAGCAGC 3'.
35 RNA was isolated from GS9L cells using the Fast Track RNA isolation kit from invitrogen using the
protocol provided. First strand cDNA synthesis was performed as follows;
1 microgram of GS9L RNA was annealed to 1 microgram of oligo151 by incubating in a volume of 6
microliter at 70C for 5' followed by cooling to room temperature. To this reaction was added
40 1.5μl 10X buffer (500mM Tris-HCl pH 8.3, 750mM KCl, 100mM MgCl₂, 5mM spermidine)
2.5μl 10mM DTT
2.5μl 10mM each dATP, dGTP, dCTP, dTTP
0.6μl 25units RNasin
2.5μl 40mM Na pyrophosphate
45 9.5μl 20 units diluted reverse transcriptase
The reaction was incubated at 42C for 1 hour. Excess oligo151 was removed by Centricon 100 spin
columns and the 5' end of the cDNA was tailed by the addition of dATP and terminal transferase. The tailed
cDNA was diluted to a final volume of 150μl in 10mM Tris-HCl, 1mM EDTA pH 7.5
PCR Reactions
50 Primary reaction 50μl
5 μl 10X buffer from Perkin Elmer Cetus GeneAmp Kit
8 μl 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
5 μl first strand GS9L cDNA prime with oligo151 and tailed
1 μl 25pMoles oligo113
55 1 μl 25 pMoles A17
1 μl 10pMoles TA17
0.25 μl 1.225 units Amplitq DNA polymerase 28.75μl water
Reaction conditions; 1 cycle 94C 1'; 50C 2'; 72C 40' then 40 cycles of 94C 1'; 50C 1'30"; 72C 2'

Prep scale secondary reaction

60μl 10Xbuffer

96μl 1.25mM each stock of dATP, dCTP, dGTP, and dTTP

6μl primary PCR reaction

5 12μl 400pMoles oligo74

12μl 300pMoles A17

3μl 15 units Amplitaq DNA polymerase

411μl water

Reaction conditions 94C 1'; 55C 2'; 72C 2' 30 cycles

10 The PCR product was concentrated by Centricon 100 spin columns, and digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform E. coli XL-I blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method. The base sequence is shown in Fig. 1.

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Claims

1. Substantially pure, biologically active glioma-derived growth factor.
2. The glioma-derived growth factor of Claim 1 wherein the growth factor is derived from mammalian
- 20 glioma cells.
3. The glioma-derived growth factor of Claim 1 wherein the factor is isolated from GS-9L cells.
4. The glioma-derived growth factor of Claim 1 wherein the growth factor is derived from human glioma cells.
5. The glioma-derived growth factor of Claim 1 wherein the factor is mitogenic for mammalian vascular
- 25 endothelial cells.
6. The glioma-derived growth factor of Claim 1 wherein the factor exists in at least three micro-heterogeneous forms with molecular masses of about 48,000, 46,000 and 44,000 daltons.
7. The glioma-derived growth factor of Claim 7 wherein the factor exists as a homodimer with each monomeric unit having a molecular mass of approximately 21,000 daltons.
- 30 8. A process for the isolation of glioma-derived growth factor in substantially pure form which comprises the sequence of steps of:
 - a. isolation of conditioned growth media;
 - b. cation exchange chromatography with adsorption on carboxymethyl Sephadex C-50 exchange resin at pH 6.0 followed by elution with 0.05 sodium phosphate pH 6.0 containing 0.6 M NaCl;
 - 35 c. lectin affinity chromatography with adsorption on concanavalin A agarose resin at pH 6.0 followed by elution with 0.05 M sodium acetate, pH 6.0, containing 1 mM CaCl₂, 1 mM MnCl₂, 0.15 M NaCl and 0.5 M alpha-methyl mannoside;
 - d. cation exchange high performance liquid chromatography with adsorption on Polyaspartic Acid WCX (Nest) followed by elution with about 0% to about 0.75 M NaCl; and
 - 40 e. reverse phase high performance liquid chromatographic purification of the glioma-derived growth factor on Vydac C₄ silica based HPLC column.
9. The process of Claim 11 for the isolation from GS-9L cell culture fluid.
10. A tissue repairing pharmaceutical composition comprising a pharmaceutical carrier and an effective tissue repairing amount of the purified glioma-derived growth factor of Claim 1.
- 45 11. The use of the glioma-derived growth factor of Claim 1, for the preparation of a medicament useful for promoting tissue repair.
12. A method for the stimulation of growth of vascular endothelial cells which comprises treating a sample of the desired endothelial cells in a nutrient medium with glioma-derived growth factor at a concentration of about 0.1-100 ng/ml.
- 50 13. The method of Claim 12 wherein cells from a vascular explant are grown on the surface of a synthetic polymeric vessel for implanting in the host.
14. The method of Claim 12 wherein endothelial cells form a vascular explant are grown on the interior surface of a biocompatible tubular mesh support and smooth muscle cells are grown on the external surface of the tubular mesh support for implanting in the host.
- 55 15. A method for preparing synthetic vessels comprising treating synthetic polymeric vessels with glioma-derived growth factor.
16. The use of glioma-derived growth factor for the preparation of a medicament useful for stimulation of the growth of vascular endothelial cells in vivo.

17. A purified and isolated DNA sequence encoding for biologically active glioma-derived growth factor.

18. The DNA sequence of claim 17 wherein the said sequence is a recombinant DNA.

19. A purified and isolated DNA sequence encoding biologically active glioma-derived growth factor comprising at least the nucleotide sequence as shown in the Figure and any subunits which encode
5 biologically active glioma-derived growth factor.

20. A recombinant glioma-derived growth factor comprising at least the amino acid sequence as shown in the Figure and any microheterogeneous or truncated forms thereof.

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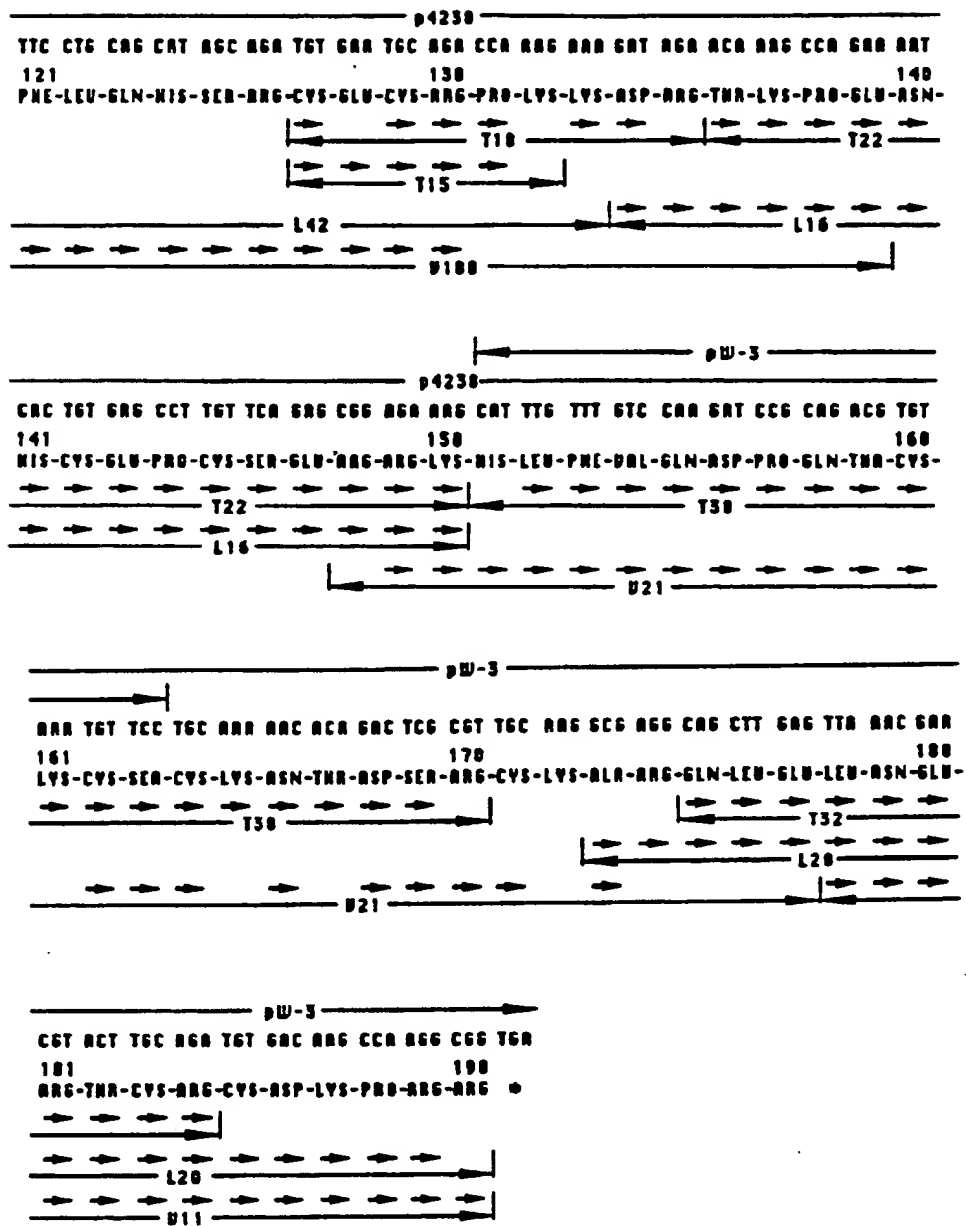
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FIGURE (cont.)





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 90 30 5637

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
X	SCIENCE. vol. 219, 1983, LANCASTER, PA US pages 983 - 985; SENGHER, D.R. et al.: "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid." * the whole document *	1	C12N15/16 C12P21/02 C07K13/00 A61K37/24
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, no. 4, February 1990, WASHINGTON US pages 1323 - 1327; CONN, G. et al.: "Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma derived cell line." * the whole document *	1-9	
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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 09 AUGUST 1990	Examiner ANDRES S.M.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application I : document cited for other reasons & : member of the same patent family, corresponding document			